REMARKS/ARGUMENTS

Claims 2, 7, 9-11 and 13 have been amended. Claims 1-4, 7-33 and 36-40 are pending in this application.

New claim 40 has been added to provoke an interference with US 6,586,583. Support for claim 40 may be found throughout the specification for example, page 5, lines 8-10 and 13-15, page 6 lines 1-11, page 13 lines 9-22, page 19 lines 14-19, and page 20 line 8 to page 21 line 12, where a plant expressible promoter or a truncated version of that promoter (i.e., a segment) is disclosed. Note that the four segments recited in claim 40 are at least "24 contiguous nucleotides selected from nucleotides 1-1532 of SEQ ID NO:2" as described on page 5 lines 13-15 of the specification.

Claims 2, 7, 9-11 and 13 have been amended to specify that the nucleotide sequence or DNA molecule is characterized as having transcriptional regulatory activity. Support for this amendment may be found throughout the specification, for example on page 13 lines 9-12. Claim 7 has also been amended to include hybridisation conditions. Support for this amendment may be found on page 13 lines 4-7, page 26 line 22 to page 27 line 3, and page 25 line 22 to page 26 line 1.

Applicant acknowledges the Examiner finding that claims 1-4, 8-9, 11-29, 32-33 and 36-39 are allowable.

Objection under 35 U.S.C. 112

Claims 7, 10 and 30-31 were rejected under 35 U.S.C. 112, second paragraph, as allegedly indefinite because the claims recite numbers without identifying them as nucleotide positions. Applicant has amended claims 7 and 10 to recite the terms "nucleotides 1-1532" and "nucleotides 1752-2382" respectively. Applicant submits that these claims, as well as dependant claims 30-31, are clear and unambiguous.

Withdrawal of the rejection under 35 U.S.C. 112, second paragraph, is requested.

Objection under 35 U.S.C. 102(b)

Examiner rejected claims 2 and 7 under 35 U.S.C. 102(b), alleging that the subject matter of the claims is anticipated by products O5878 and O3378 of the Sigma Chemical Company Catalog. Applicant respectfully disagrees with the Examiner's rejection.

Amended claim 7 requires that the nucleotide sequence hybridise to the sequence of nucleotides 1-1532 of SEQ ID NO:2 or a complement thereof, and that the nucleotide sequence have transcriptional regulatory activity. Furthermore, conditions for hybridisation and washing are recited in the claim. Similarly, claim 2 has been amended to recite that the DNA molecule has transcriptional regulatory activity.

Therefore, Applicant submits that claims 2 and 7 are not anticipated by products O5878 and O3378 of the Sigma Catalog, and withdrawal of the rejection under 35 U.S.C. 102(b) is requested.

Potential Interference

The Examiner noted that nucleotides 73-4655 of SEQ ID NO:2 are 100% identical to SEQ ID NO:20 of Vierling, Jr., US 6,586,583 (the '583 patent) and may support interference proceedings involving the '583 patent and the present application. In response, Applicant provides the following comments.

1) Review of the '583 patent and related patents (US 5,866,695 and US 5,840,558) issuing from earlier filed parent applications reveals that SEQ ID NO:20, cited by Examiner , was first disclosed in the '583 patent. This nucleotide sequence was not disclosed in the earlier filed applications as only SEQ ID NOS:1-19 were present in US 5,866,695, and SEQ ID NOS:1-17 were present in US 5,840,558.

2) The '583 patent was filed on December 9, 1998 which is the effective filing date of Vierling's claims directed to SEQ ID NO:20.

3) The present application, disclosing SEQ ID NO:2, was filed on September 29, 1997 which is more than one year prior to the filing date of the '583 patent.

4) The sequence information contained in SEQ ID NO:2 was disclosed by Applicant in November 1997 in *The Plant Journal* (vol 12, pp. 9991-9998; copy enclosed), the NCBI database under accession number AF014502 (copy enclosed), and the Medline database under reference number 98079236 (or the PubMed database under reference number 9418041; copy enclosed) which establish that SEQ ID NO:2 was publicly known more than one year prior to the filing date of the '583 patent.

5) The *Plant Journal* paper is cited on the front page of the '583 patent.

6) An interference should have been declared while the '583 patent was co-pending with the present application in the U.S. Patent and Trademark Office.

In view of the above, claim 7 of the '583 patent has been copied herein as claim 40 of the present application. In accordance with 37 CFR § 1.607, Applicant requests that an interference be declared between the '583 patent and the present application. The proposed count is (i) claim 7 of the '583 patent or (ii) claims 2 and 40 of the present application. The claims are presented less than one year from the issue date of the '583 patent (i.e., July 1, 2003).

Since Vierling's claims (including claim 7) recite SEQ ID NO:20 as a limitation, the effecttive filing date of the '583 patent is December 9, 1998. The effective date of the present application is September 29, 1997. Therefore, Applicant should be named the senior party in the interference.

It is respectfully submitted that the above-identified application is now in a condition for allowance, therefore favourable reconsideration and prompt allowance of these claims are respectfully requested. Should the Examiner believe that anything further is desirable in order to place the application in better condition for allowance, the Examiner is invited to contact the applicant's undersigned attorney at the telephone number listed below.

Respectfully submitted

By 

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Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

1: AF014502. Glycine max seed ...[gi:2342665]

```
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ACCESSION   AF014502
VERSION     AF014502.1  GI:2342665
KEYWORDS    .
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  ORGANISM  Glycine max
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            rosids; eurosids I; Fabales; Fabaceae; Papilionoideae; Phaseoleae;
            Glycine.
REFERENCE   1  (bases 1 to 4700)
  AUTHORS   Gijzen,M.
  TITLE     A deletion mutation at the ep locus causes low seed coat peroxidase
            activity in soybean
  JOURNAL   Plant J. 12 (5), 991-998 (1997)
  MEDLINE   98079236
   PUBMED   9418041
REFERENCE   2  (bases 1 to 4700)
  AUTHORS   Gijzen,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-JUL-1997) Research Branch, Agriculture Canada, 1391
            Sandford Street, London, ON N5V 4T3, Canada
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polyA_site 4700

ORIGIN

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1: Plant J. 1997 Nov;12(5):991-8.

Related Articles, Link

Full text

A deletion mutation at the ep locus causes low seed coat peroxidase activity in soybean.

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The Ep locus severely affects the amount of peroxidase enzyme in soybean seed coats. Plants containing the dominant Ep allele accumulate large amounts of peroxidase in the hourglass cells of the sub-epidermis. Homozygous recessive epep genotypes do not accumulate peroxidase in the hourglass cells and are much reduced in total seed coat peroxidase activity. To isolate the gene encoding the seed coat peroxidase and to determine whether it corresponds to the Ep locus, a cDNA library was constructed from developing seed coats and an abundant 1.3 kb peroxidase transcript was cloned. The corresponding structural gene was also isolated from a genomic library. Sequence analysis shows that the seed coat peroxidase is translated a a 352 amino acid precursor protein of 38 kDa. Processing of a putative 26 amino acid signal sequence results in a mature protein of 326 residues with a calculated mass of 35 kDa and a pI of 4.4. Using probes derived from the cDNA, genomic DNA blot hybridization and polymerase chain reaction analysis detected polymorphisms that distinguished EpEp and epep genotypes. Co-segregation of the polymorphisms in an F2 population from a cross of EpEp and epep plants shows that the Ep locus encodes the seed coat peroxidase protein. Comparison of Ep and ep alleles indicates that the recessive gene lacks 87 bp of sequence encompassing the translation start codon. Analysis by RNA blot hybridization shows that epep plants have drastically reduced amounts of peroxidase transcript compared with EpEp plants. The peroxidase mRNA is abundant in seed coat tissues of EpEp plant during the late stages of seed maturation, and could also be detected in root tissues, but not in the flower, embryo, pod or leaf. The results indicate that th lack of peroxidase accumulation in seed coats of homozygous recessive epep plants is due to a mutation of the structural gene that reduces transcript abundance.

The Plant Journal (1997) 12(5), 991–998

A deletion mutation at the *ep* locus causes low seed coat peroxidase activity in soybean

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Summary

The *Ep* locus severely affects the amount of peroxidase enzyme in soybean seed coats. Plants containing the dominant *Ep* allele accumulate large amounts of peroxidase in the hourglass cells of the sub-epidermis. Homozygous recessive *epep* genotypes do not accumulate peroxidase in the hourglass cells and are much reduced in total seed coat peroxidase activity. To isolate the gene encoding the seed coat peroxidase and to determine whether it corresponds to the *Ep* locus, a cDNA library was constructed from developing seed coats and an abundant 1.3 kb peroxidase transcript was cloned. The corresponding structural gene was also isolated from a genomic library. Sequence analysis shows that the seed coat peroxidase is translated as a 352 amino acid precursor protein of 38 kDa. Processing of a putative 26 amino acid signal sequence results in a mature protein of 326 residues with a calculated mass of 35 kDa and a pI of 4.4. Using probes derived from the cDNA, genomic DNA blot hybridization and polymerase chain reaction analysis detected polymorphisms that distinguished *EpEp* and *epep* genotypes. Co-segregation of the polymorphisms in an F_2 population from a cross of *EpEp* and *epep* plants shows that the *Ep* locus encodes the seed coat peroxidase protein. Comparison of *Ep* and *ep* alleles indicates that the recessive gene lacks 87 bp of sequence encompassing the translation start codon. Analysis by RNA blot hybridization shows that *epep* plants have drastically reduced amounts of peroxidase transcript compared with *EpEp* plants. The peroxidase mRNA is abundant in seed coat tissues of *EpEp* plants during the late stages of seed maturation, and could also be detected in root tissues, but not in the flower, embryo, pod or leaf. The results indicate that the lack of peroxidase accumulation in seed coats of homozygous recessive *epep* plants is due to a mutation of the structural gene that reduces transcript abundance.

Introduction

Peroxidases are enzymes catalysing oxidative reactions that use H_2O_2 as an electron acceptor. These enzymes are widespread and occur ubiquitously in plants as isozymes that may be distinguished by their isoelectric points. Plant peroxidases contribute to the structural integrity of cell walls by functioning in lignin biosynthesis and suberization, and by forming covalent cross-linkages between extensin, cellulose, pectin and other cell wall constituents (Campa, 1991). Peroxidases are also associated with plant defence responses and resistance to pathogens (Bowles, 1990; Moerschbacher, 1992).

Peroxidases are good targets for isozyme analysis in genetic studies since these enzymes usually have wide specificity and are able to oxidize many different colorimetric organic substrates. In an analysis of soybean seeds, Buttery and Buzzell (1968) showed that the amount of peroxidase activity present in seed coats may vary substantially among different cultivars. The presence of a single dominant gene *Ep* causes a high seed coat peroxidase phenotype (Buzzell and Buttery, 1969). Homozygous recessive *epep* plants are 100-fold lower in seed coat peroxidase activity. This results from a reduction in the amount of peroxidase enzyme present, primarily in the hourglass cells of the sub-epidermis (Gijzen *et al.*, 1993). In plants carrying the *Ep* gene, peroxidase is heavily concentrated in these cells. The hourglass cells, also called osteosclereids, form a highly differentiated cell layer with thick, elongated secondary walls and large intercellular spaces (Baker *et al.*, 1987). Hourglass cells develop between the epidermal macrosclereids and the underlying articulated parenchyma, and are a prominent feature of seed coat anatomy at full maturity. The cytoplasm exudes from the hourglass cells upon imbibition of water and a distinct peroxidase isozyme constitutes 5–10% of the total soluble protein in *EpEp* seed coats (Gillikin and Graham, 1991). It is not known why the hourglass cells accumulate large amounts of peroxidase, but the sheer abundance and relative purity of the enzyme in soybean hulls is significant because peroxidases are versatile enzymes with many commercial and industrial applications.

In this study, cDNA and genomic clones encoding the seed coat peroxidase are identified and characterized. Restriction fragment length polymorphism and PCR analysis of *EpEp* and *epep* genotypes and F_2 progeny reveals that the *Ep* locus corresponds to the seed coat peroxidase structural gene. The naturally occurring recessive *ep* allele harbours an 87 bp deletion at the 5′ end of the gene. This mutation causes a reduction in peroxidase mRNA prevalence and results in the low activity phenotype associated with the *ep* gene.

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Results

The seed coat peroxidase cDNA and structural gene show similarities to other plant peroxidase genes

To isolate the seed coat peroxidase transcript, a cDNA library was constructed from developing seed coat tissue of the *EpEp* cultivar Harosoy 63. The primary library contained 10^6 recombinant plaque-forming units and was amplified prior to screening. A degenerate 17-mer oligonucleotide corresponding to the conserved active site domain of plant peroxidases was used to probe the library. In a screening of 10 000 plaque-forming units, 12 positive clones were identified. The cDNA insert size of the clones ranged from 0.5–2.5 kb, but six clones shared a common insert size of 1.3 kb. These six clones were chosen for further characterization since the 1.3 kb insert size matched the expected peroxidase transcript size. Sequence analysis of the six clones showed that they contained identical cDNA transcripts encoding a peroxidase and that each resulted from an independent cloning event since the junction between the cloning vector and the transcript was different in all cases.

Since it was not clear that the entire 5′ end of the cDNA transcript was complete in any of the cDNA clones isolated, the structural gene corresponding to the seed coat peroxidase was isolated from a Harosoy 63 genomic library. A partial *Bam*HI digest of genomic DNA was used to construct the library and more than 10^6 plaque-forming units were screened using the cDNA probe. A positive clone containing a 17 kb insert was identified and a 4.8 kb region encoding the peroxidase was sequenced. The genomic sequence matched the cDNA sequence except for three introns encoded within the gene. The genomic sequence also revealed two additional translation start codons, beginning 1 bp and 10 bp upstream from the 5′ end of the longest cDNA transcript isolated. Figure 1 shows the deduced cDNA sequence. The open reading frame of 1056 bp encodes a 352 amino acid protein of 38 106 Da. A haem-binding domain, a peroxidase active site signature sequence, and seven potential N-glycosylation sites were identified from the deduced amino acid sequence. The first 26 amino acid residues conform to a membrane-spanning domain. Cleavage of this putative signal sequence releases a mature protein of 326 residues with a mass of 35 377 Da and an estimated pI of 4.4.

Figure 2 illustrates the relationship between the soybean seed coat peroxidase and other selected plant peroxidases. The soybean sequence is most closely related to four peroxidase cDNAs isolated from alfalfa, sharing from 65–67% identity at the amino acid level with the alfalfa proteins. When compared with other plant peroxidases, soybean seed coat peroxidase exhibits

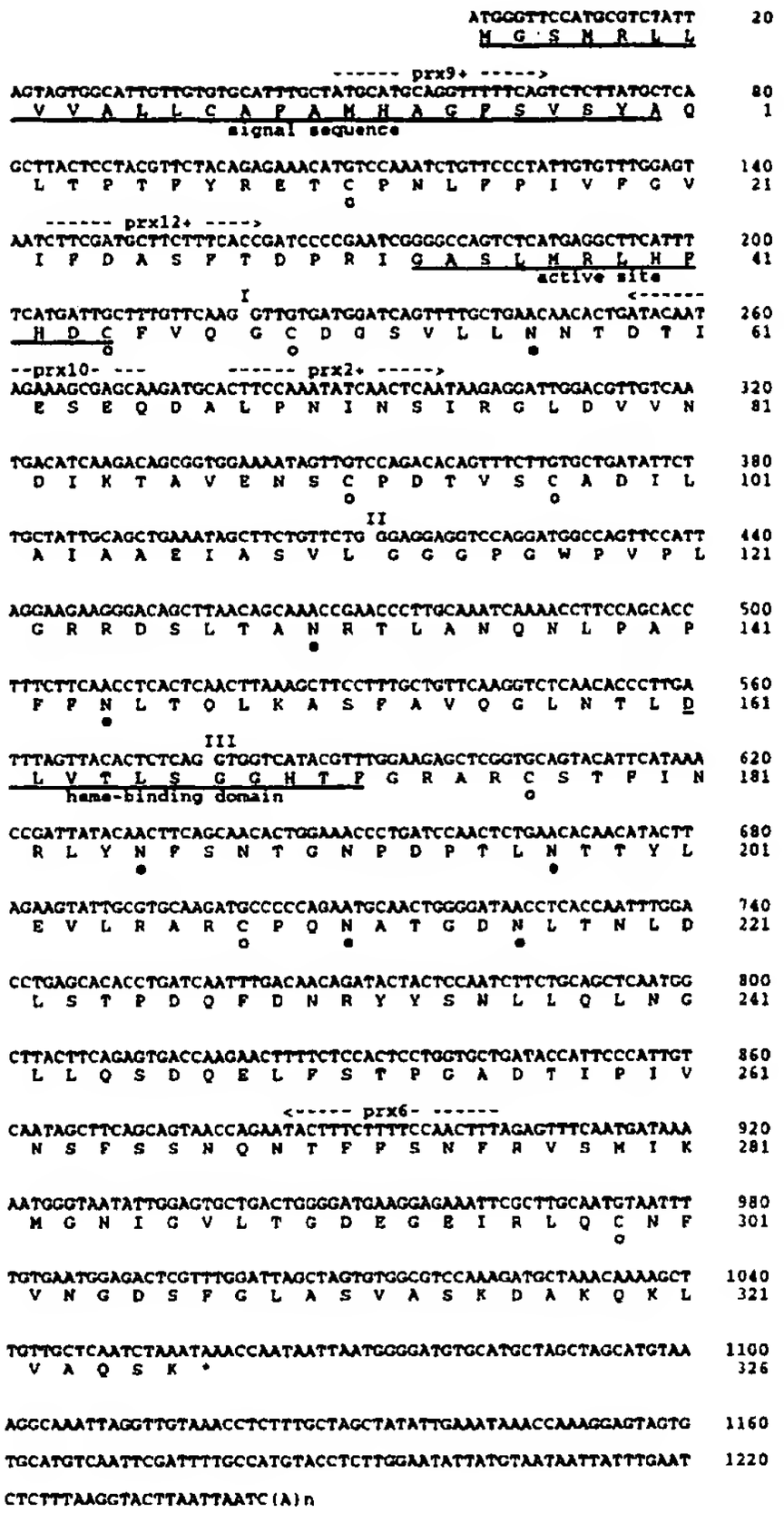


Figure 1. The cDNA and deduced amino acid sequence of soybean seed coat peroxidase.
Nucleotides are numbered by assigning +1 to the first base of the ATG start codon; amino acids are numbered by assigning +1 to the N-terminal Gln residue after cleavage of the putative signal sequence. The eight conserved cysteine residues are indicated with open circles (O) and the seven potential N-glycosylation sites with closed circles (●). The N-terminal signal sequence, the region of the active site, and the haem-binding domain are underlined. The numerals I, II and III placed directly above single nucleotide gaps in the sequence indicate the three intron splice positions. The target site and direction of five different PCR primers are shown with dotted lines above the nucleotide sequence. An asterix (*) marks the translation stop codon. The first 10 bp were deduced from the sequence of the genomic clone and the remainder was directly sequenced from the cDNA.

from 60–65% identity with poplar (D30653 and D30652) and flax (L07554), 50–60% identity with horseradish (M37156), tobacco (D11396) and cucumber (M91373), and 49% identity with barley (L36093), wheat (X85228) and tobacco (L02124) peroxidases.

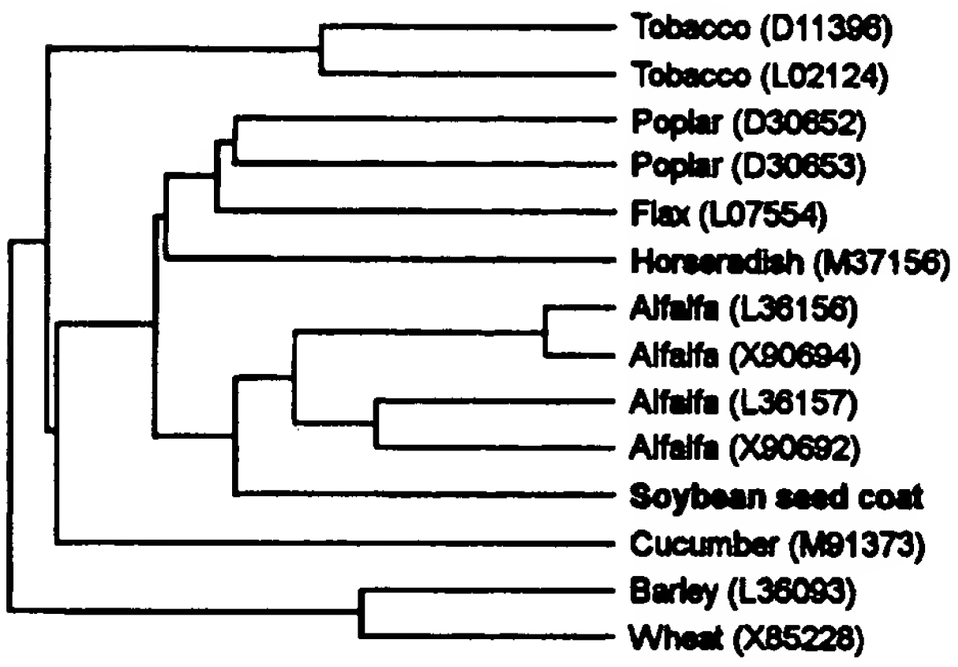


Figure 2. Comparison of soybean seed coat peroxidase and other selected plant peroxidases.
A dendogram from the alignment of the amino acid sequence of soybean seed coat peroxidase and 13 other plant peroxidases is shown. The GenBank accession numbers are provided next to the name of the plant from which the peroxidase was isolated. The accession number for the soybean sequence is AFD14502. Alignments were performed using the CLUSTAL program of the PCGENE version 6.85 software package (InteliGenetics, Mountain View, CA).

DNA blot analysis using the seed coat peroxidase cDNA probe reveals restriction fragment length polymorphisms between EpEp *and* epep *genotypes*

Genomic DNA blots of OX347 (*EpEp*) and OX312 (*epep*) plants were hybridized with ^{32}P-labelled cDNA to estimate the copy number of the seed coat peroxidase gene and to determine whether this locus is polymorphic between the two genotypes. Figure 3 shows the hybridization patterns after digestion with *Bam*HI, *Xba*I and *Sac*I. Restriction fragment length polymorphisms are clearly visible in the *Bam*HI and *Sac*I digestions. The *Bam*HI digestion produced a strongly hybridizing 17 kb fragment and a faint 3.4 kb fragment in the *EpEp* genotype. The 3.4 kb *Bam*HI fragment is visible in the *epep* genotype but the 17 kb fragment has been replaced by a signal at > 20 kb. The *Sac*I digestion resulted in detection of three fragments in *EpEp* and *epep* plants. At least two fragments were expected here since the cDNA sequence has a *Sac*I site within the open reading frame. However, the smallest and most strongly hybridizing of these fragments is 5.2 kb in *EpEp* plants and 4.9 kb in *epep* plants. Digestion with *Xba*I produced hybridizing fragments of 14 kb and 7.8 kb for both genotypes, with the larger fragment showing a stronger signal.

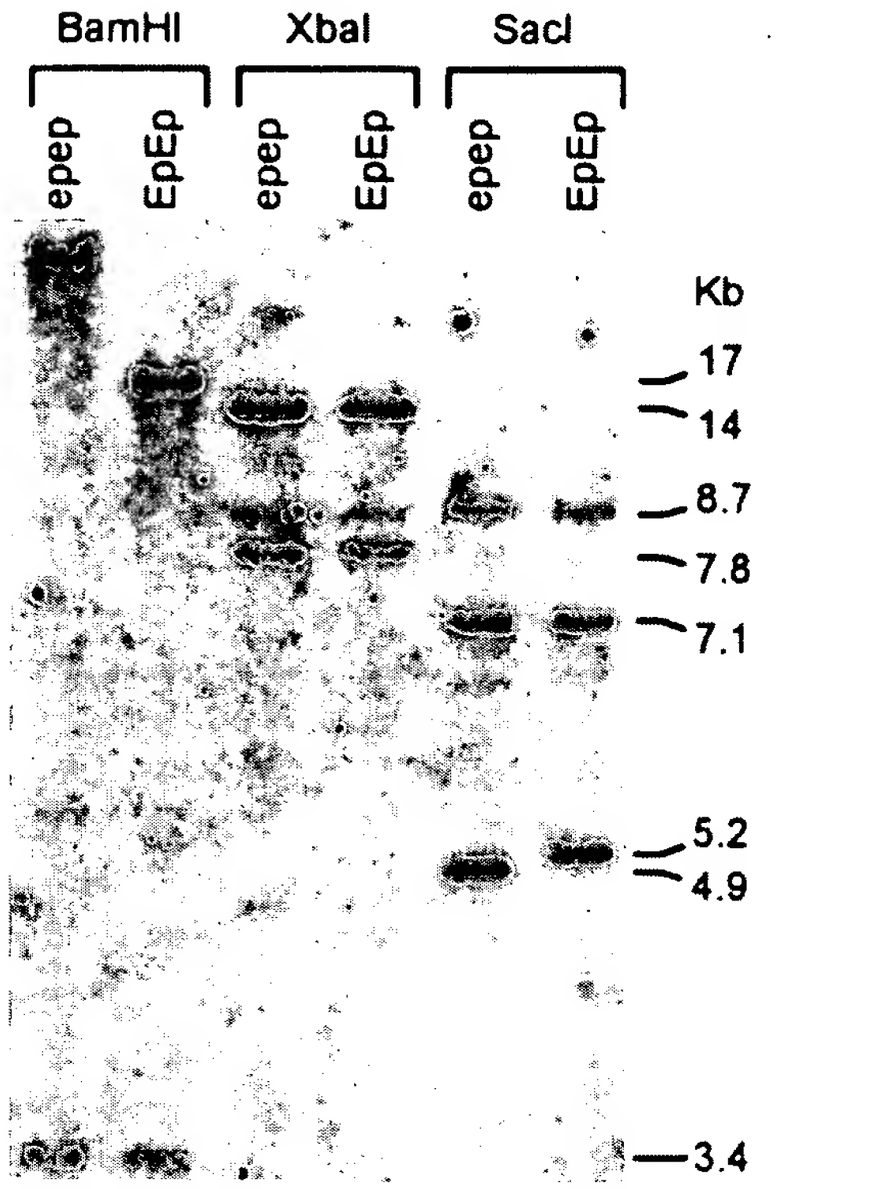


Figure 3. Restriction fragment length polymorphisms between *EpEp* and *epep* genotypes using the seed coat peroxidase cDNA as probe.
Genomic DNA of soybean lines OX312 (*epep*) and OX347 (*EpEp*) was digested with restriction enzyme, separated by electrophoresis in a 0.5% agarose gel, transferred to nylon, and hybridized with ^{32}P-labelled cDNA encoding the seed coat peroxidase. The size of the hybridizing fragments was estimated by comparison with standards and is indicated on the right.

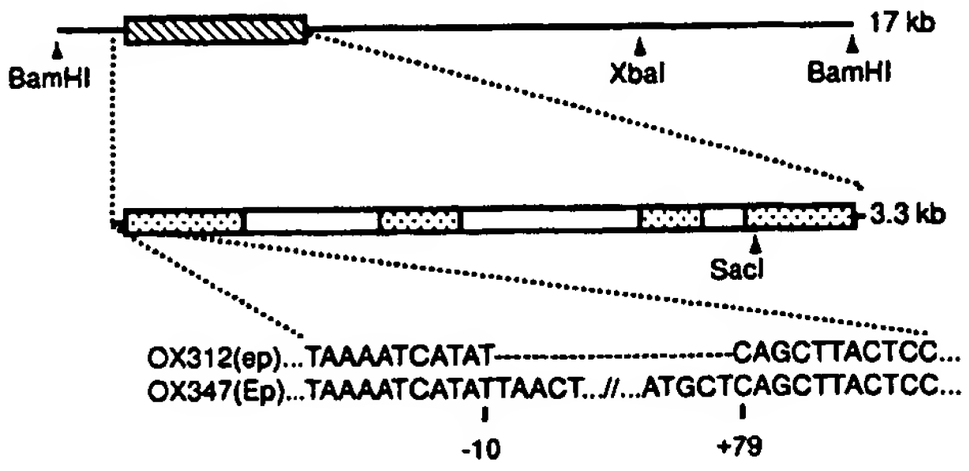


Figure 4. Structure of the *Ep* locus
A 17 kb fragment including the *Ep* locus is illustrated schematically. A 3.3 kb portion of the gene is enlarged and exons and introns are represented by shaded and open boxes, respectively. The final enlargement of the 5' region shows the location and DNA sequence around the 87 bp deletion occurring in the *ep* allele of soybean line OX312. Nucleotides are numbered by assigning +1 to the first base of the ATG start codon.

A deletion mutation occurs in the recessive ep *locus*

The structural gene encoding the seed coat peroxidase is schematically illustrated in Figure 4. The 17 kb *Bam*HI fragment encompassing the gene includes 1.5 kb of sequence upstream from the translation start codon, three introns of 631 bp, 1030 bp and 263 bp, and 12 kb of sequence downstream from the polyadenylation site. The arrangement of four exons and three introns and the position of introns within the sequence is similar to that described for other plant peroxidases (Osakabe *et al.*, 1995; Simon, 1992).

Primers were designed from the DNA sequence to compare *EpEp* and *epep* genotypes by PCR analysis. Figure 5 shows PCR amplification products from four different

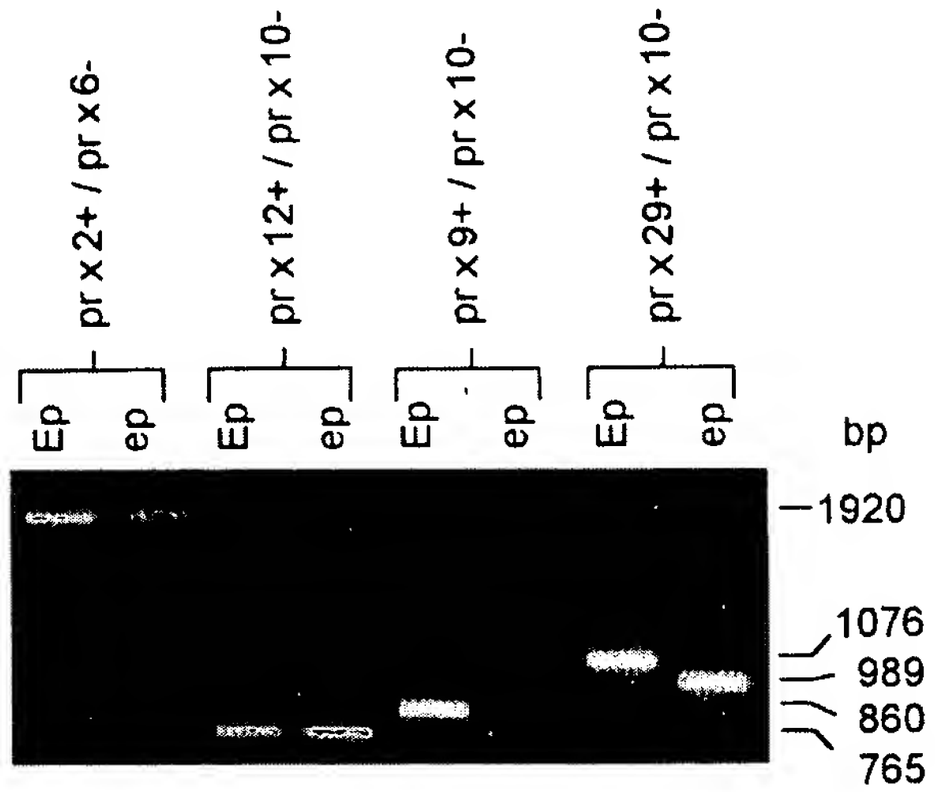


Figure 5. PCR analysis of *EpEp* and *epep* genotypes using primers derived from the seed coat peroxidase cDNA
Genomic DNA from soybean lines OX312 (*epep*) and OX347 (*EpEp*) was used as template for PCR analysis with four different primer sets. Amplification products were separated by electrophoresis through a 0.8% agarose gel and visualized under UV light after staining with ethidium bromide. Genotype and primer combinations are indicated at the top. The size in base pairs of the amplified DNA fragments are indicated on the right.

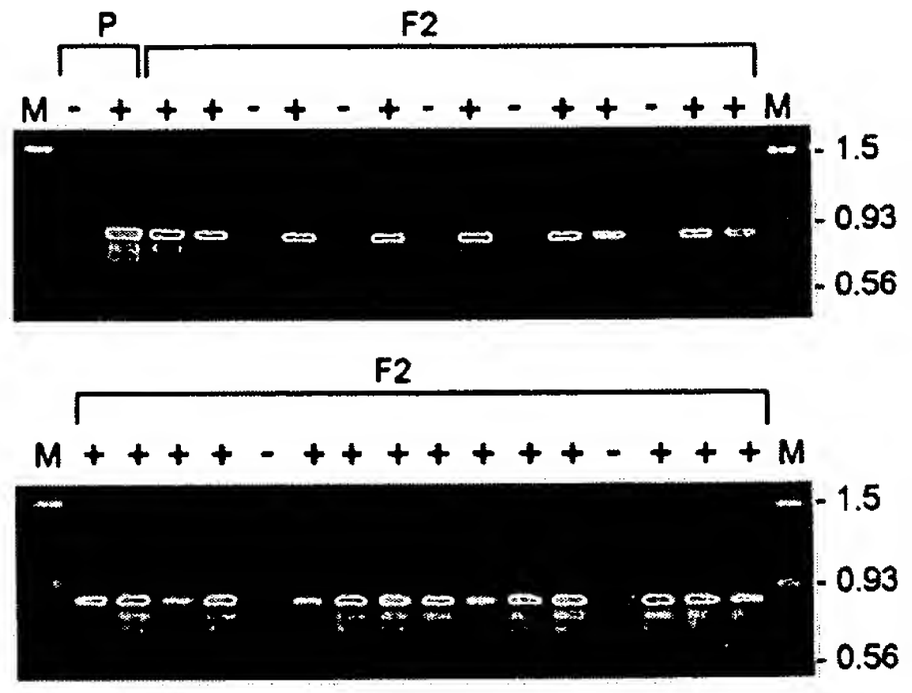


Figure 6. PCR analysis of an F_2 population from a cross of *EpEp* and *epep* genotypes.
Genomic DNA was used as template for PCR analysis of the parents (P) and 30 F_2 individuals. The cross was derived from the soybean lines OX312 (*epep*) and OX347 (*EpEp*). Plants were self-pollinated and seeds were collected and scored for seed coat peroxidase activity. The symbols (–) and (+) indicate low and high seed coat peroxidase activity, respectively. Primers prx9+ and prx10– were used in the amplification reactions, as described in Experimental procedures. Products were separated by electrophoresis through a 0.8% agarose gel and visualized under UV light after staining with ethidium bromide. The migration of molecular markers and their corresponding size in kb is also shown (lanes M).

primer combinations using OX312 (*epep*) and OX347 (*EpEp*) genomic DNA as template. The primer annealing site for prx29+ begins 182 bp upstream from the ATG start codon; the remaining primer sites are shown in Figure 1. Amplification with primers prx2+ and prx6–, and with prx12+ and prx10– produced the expected products of 1.9 kb and 860 bp, respectively, regardless of the *Ep*/*ep* genotype of the template DNA. However, PCR amplification with primers prx9+ and prx10–, and with prx29+ and prx10– generated the expected products only when template DNA was from plants carrying the dominant *Ep* allele. When template DNA was from an *epep* genotype, no product was detected using primers prx9+ and prx10– and a smaller product was amplified with primers prx29+ and prx10–. The products resulting from amplification of OX312 or OX347 template DNA with primers prx29+ and prx10– were directly sequenced and compared. The polymorphism is due to an 87 bp deletion occurring within this DNA fragment in OX312 plants, as shown in Figure 4. This deletion begins 9 bp upstream from the translation start codon and includes 78 bp of sequence at the 5′ end of the open reading frame, including the prx9+ primer annealing site.

To test whether this deletion mutation co-segregates with the seed coat peroxidase phenotype, genomic DNA from an F_2 population segregating at the *Ep* locus was amplified using primers prx9+ and prx10–, and F_3 seed was tested for seed coat peroxidase activity. Figure 6 shows the results from this analysis. Of the 30 F_2 individuals tested, all 23 that were high in seed coat peroxidase activity produced the expected 860 bp PCR amplification product. The remaining seven F_2 individuals with low seed coat peroxidase activity produced no detectable PCR amplification products.

To determine whether the OX312 (*epep*) and OX347 (*EpEp*) breeding lines are representative of soybean cultivars that differ in seed coat peroxidase activity, several cultivars were tested by PCR analysis using primer combinations targeted to the *Ep* locus. Figure 7 shows results from this analysis of six different soybean cultivars, three each of the homozygous dominant *EpEp* and recessive *epep* genotypes. As observed with OX312 and OX347, amplification products of the expected size were produced with primers prx12+ and prx10– regardless of the genotype, whereas *epep* genotypes yielded no product with primers prx9+ and prx10– or a smaller fragment with primers prx29+ and prx10–.

Seed coat peroxidase mRNA levels are reduced in epep *plants*

The seed coat peroxidase mRNA levels were determined by hybridizing RNA gel blots with radiolabelled cDNA probe. Figure 8 illustrates the transcript abundance in various tissues of *epep* and *EpEp* plants. The mRNA accumulated to high levels in seed coat tissues of *EpEp* plants, especially in the later stages of development when whole-seed fresh weight exceeded 50 mg. Low levels of transcript could also be detected in root tissues but not in the flower,

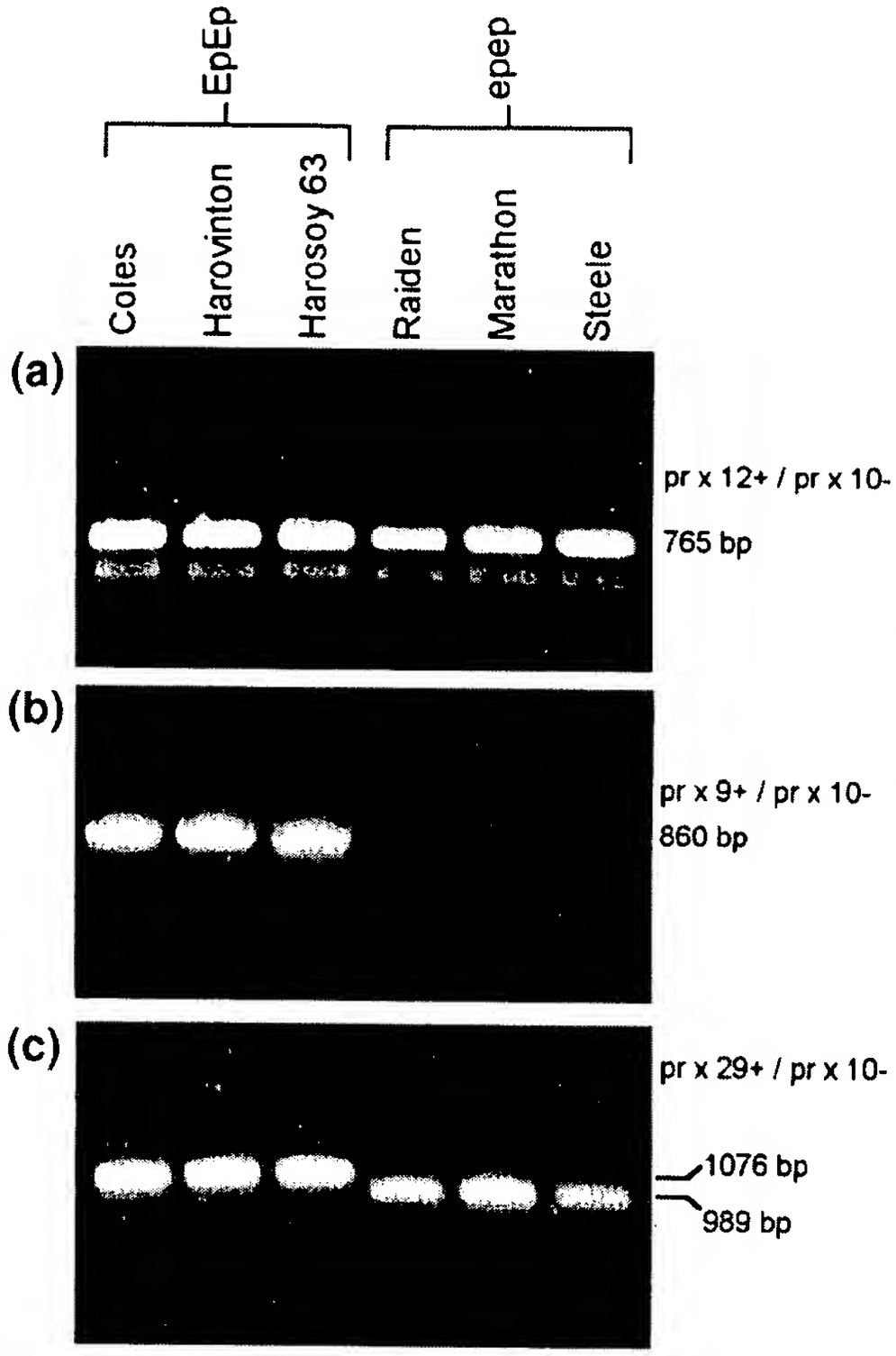
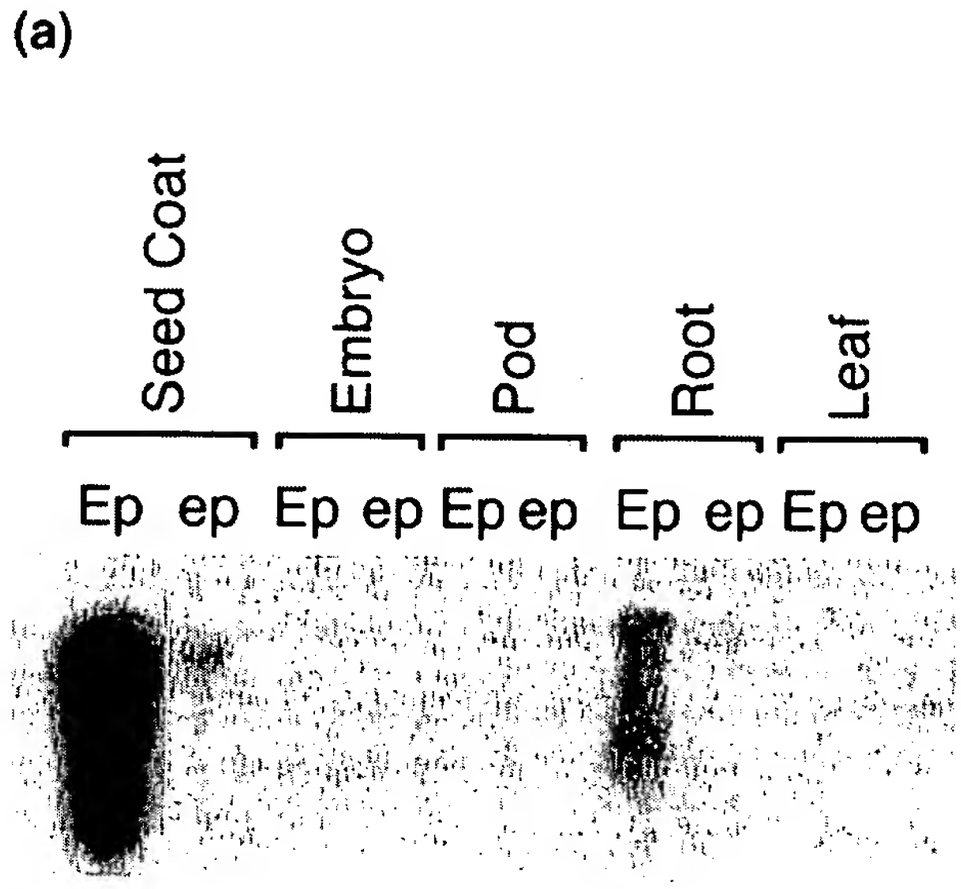


Figure 7. PCR analysis of six different soybean cultivars with primers derived from the seed coat peroxidase cDNA sequence.
Genomic DNA was used as template for PCR analysis of three *EpEp* cultivars and three *epep* cultivars. Primers used in the amplification reactions and the size of the DNA product are indicated on the right. Products were separated by electrophoresis through a 0.8% agarose gel and visualized under UV light after staining with ethidium bromide.
(a) Forward and reverse primers are downstream from the deletion.
(b) Forward primer anneals to site within the deletion.
(c) Primers span the deletion.



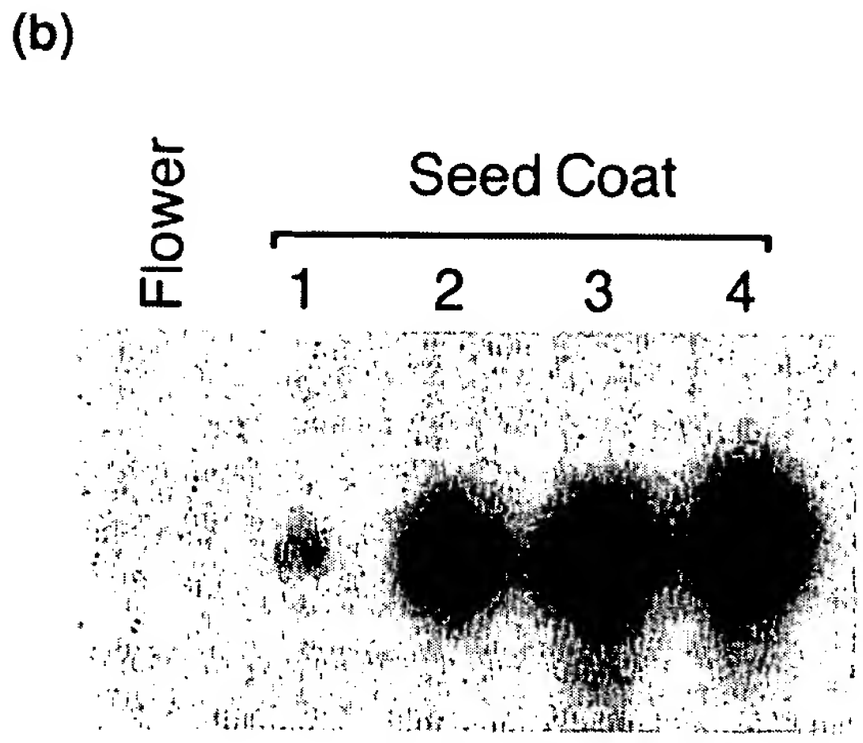


Figure 8. Accumulation of peroxidase mRNA in tissues of *EpEp* and *epep* plants.
A 10 μg aliquot of total RNA from each sample was separated by electrophoresis through a 1% agarose gel containing formaldehyde, blotted, and probed as described in Experimental procedures. RNA was visualized after electrophoresis by ethidium bromide fluorescence to ensure that equivalent amounts were loaded for each of the samples.
(a) A comparison of peroxidase transcript abundance in cultivars Harosoy 63 (*Ep*) or Marathon (*ep*). Seed and pod tissues were sampled at a late stage of development corresponding to a whole-seed fresh weight of 250 mg. Root and leaf tissue was from 6-week-old plants. Autoradiograph exposed for 96 h.
(b) Developmental expression of peroxidase in cultivar Harosoy 63 (*Ep*). Flowers were sampled immediately after opening. Seed coat tissues were sampled at four stages of development corresponding to a whole-seed fresh weight of: lane 1, 50 mg; lane 2, 100 mg; lane 3, 200 mg; lane 4, 250 mg. Autoradiograph exposed for 20 h.

embryo, pod or leaf. The transcript could also be detected in seed coat and root tissues of *epep* plants but in drastically reduced amounts compared with the *EpEp* genotype.

Discussion

There are several naturally occurring mutations affecting soybean seed protein content and coloration that have been described at the molecular level. These mutations include a 5′ deletion in the cgy_1 gene encoding the α-subunit of β-congycinin (Ladin *et al.*, 1984), insertion of a 3.4 kb transposon-like element in the seed lectin *le* gene (Vodkin *et al.*, 1983), translational frame shift mutations affecting the gycinin gy_4 gene (Scallon *et al.*, 1987) and the Kunitz trypsin inhibitor *ti* gene (Jofuku *et al.*, 1989), and a translocation interrupting the glycinin gy_3 gene (Cho *et al.*, 1989). Structural gene rearrangements may also have unpredictable consequences on gene expression. The *I*

locus controlling soybean seed coat coloration comprises several chalcone synthase genes that interact to produce a variety of pigmentation phenotypes (Todd and Vodkin, 1996).

Allelic variation at the *Ep* locus of soybean is naturally occurring and commercially important since this affects the amount of peroxidase enzyme contained in the seed coat. Homozygous dominant *EpEp* cultivars command a higher market value in some areas because large amounts of active peroxidase enzyme may be extracted and recovered from seed hulls. Studies of soybean seed coat peroxidase have shown this enzyme to have useful catalytic properties and a high degree of thermal stability even at extremes of pH (McEldoon *et al.*, 1995). This work describes the isolation of the seed coat peroxidase cDNA and structural gene corresponding to the *Ep* locus, and shows that the recessive *ep* allele has an 87 bp deletion mutation surrounding the translation start site.

Screening the seed coat cDNA library with a degenerate primer derived from the active site domain of plant peroxidases resulted in a relatively high frequency of positive clones. That many of these clones encoded identical cDNA molecules indicates that the corresponding mRNA is an abundant transcript in developing seed coat tissues. This is not surprising since the enzyme accumulates in the hourglass cells of the sub-epidermis and is a major component of the soluble protein fraction in seed coat extracts of high peroxidase cultivars. A partial cDNA clone corresponding to a seed coat peroxidase from the soybean cultivar Williams 82 has been reported in the Plant Gene Register (Huangpu *et al.*, 1996). This sequence is nearly identical to that described here but contains several nucleotide and amino acid differences. Nonetheless, the two sequences probably correspond to the same gene.

Previous studies on soybean seed coat peroxidase indicated that this enzyme is heavily glycosylated and that carbohydrate contributes 18% of the mass of the apo-enzyme (Gray *et al.*, 1996). The seven potential glycosylation sites identified from the amino acid sequence would accommodate the five or six *N*-linked glycosylation sites proposed by Gray *et al.* (1996). The molecular mass of the enzyme has been determined by denaturing gel electrophoresis to be 37 kDa (Gillikin and Graham, 1991; Sessa and Anderson, 1981) or 43 kDa (Gijzen *et al.*, 1993). Analysis by mass spectrometry indicated a mass of 40 622 Da for the apo-enzyme and 33 250 Da after deglycosylation (Gray *et al.*, 1996). These values are in good agreement with the mass of 35 377 Da calculated from the predicted amino acid sequence for the mature apo-protein prior to glycosylation and other modifications. The haem-binding domain encompasses residues Asp161 to Phe171 and the acid–base catalysis region from Gly33 to Cys44. The two regions are highly conserved among plant peroxidases and are centred around functional histidine residues, His169 and His40. There are eight conserved cysteine residues in the mature protein that provide for the four disulphide bridges found in other plant peroxidases and predicted from the crystal structure of peanut peroxidase (Schuller *et al.*, 1996; Welinder, 1992). Other conserved areas include residues Cys91 to Ala105 and Val119 to Leu127 that occur in or around helix D. The most divergent aspects of the seed coat peroxidase protein sequence are the carboxy- and amino-terminal regions. These sequences typically provide special targeting signals for the proper processing and delivery of the peptide chain. Although the enzyme appears to accumulate intracellularly within the hourglass cells, the exact subcellular location is not known. The carboxy-terminus is homologous to that of other vacuolar targeted peroxidases (Omann and Tyson, 1996) but is distinguished from these by its high lysine content.

Genomic DNA blots probed with the seed coat peroxidase cDNA produced two or three hybridizing fragments of varying intensity with most restriction enzyme digestions, despite the fact that several peroxidase isozymes are present in soybean. The results indicate that the seed coat peroxidase is present as a single gene that does not share sufficient homology with most other peroxidase genes to anneal under conditions of high stringency. Digestion with *Bam*HI or *Sac*I revealed restriction fragment length polymorphisms that distinguished *EpEp* and *epep* genotypes. Although the *Xba*I digestion did not produce a readily detectable polymorphism, the size of the hybridizing fragment in both genotypes was 14 kb. A 0.3 kb size difference is beyond the resolving power of the separation for fragments this large. Sequence analysis of *EpEp* and *epep* genotypes indicates that the mutant *ep* allele is missing 87 bp of sequence at the 5′ end of the structural gene. However, the 87 bp deletion cannot account for the differences observed in the RFLP analysis since the missing fragment does not include a *Bam*HI site and is much smaller than the 0.3 kb polymorphism detected in the *Sac*I digestion. Other genetic rearrangements must occur in the vicinity of the *ep* locus that lead to these polymorphisms.

Analysis of peroxidase transcript abundance by RNA blot hybridization shows that the *Ep* gene is specifically expressed in seed coat tissues during the later stages of seed maturation. The transcript could also be detected at low levels in RNA samples from the root, indicating that the *Ep* gene is weakly expressed in this tissue. This was expected since the corresponding isozyme occurs in roots of *EpEp* genotypes (Gijzen *et al.*, 1993). The reduced amounts of peroxidase mRNA present in seed coats of *epep* plants indicates that the transcriptional process and/or the stability of the resulting mRNA is severely affected. The *Ep* gene has a TATA box and a 5′ cap signal begining 47 bp and 15 bp, respectively, upstream from the translation start codon. The 87 bp deletion in the *ep* allele extends into the 5′ cap signal and therefore could interfere with

transcript processing. Regardless, any resulting transcript will not be properly translated since the AUG initiation codon and the entire amino-terminal signal sequence are deleted from the *ep* allele. Thus, the lack of peroxidase accumulation in seed coats of *epep* plants is probably due to at least two factors, i.e. greatly reduced transcript levels and ineffective translation, resulting from mutation of the structural gene encoding the enzyme.

Experimental procedures

Plant materials

All soybean (*Glycine max* [L.] Merr) cultivars and breeding lines were from the collection at Agriculture Canada, Harrow, Ontario.

Seed coat cDNA library construction and screening

High seed coat peroxidase (*EpEp*) soybean cultivar Harosoy 63 plants were grown in field plots outdoors. Pods were harvested 35 days after flowering and seeds in the mid- to late developmental stage were excised. The average fresh mass was 250 mg per seed. Seed coats were dissected and immediately frozen in liquid nitrogen. The frozen tissue was lyophilized and total RNA extracted in 100 mM Tris-HCl pH 9.0, 20 mM EDTA, 4% (w/v) sarkosyl, 200 mM NaCl, and 16 mM DTT, and precipitated with LiCl using the standard phenol/chloroform method described by Wang and Vodkin (1994). The poly(A)+ RNA was purified on oligo(dT) cellulose columns prior to cDNA synthesis, size selection, ligation into the λZAP Express vector, and packaging according to instructions (Stratagene). A degenerate oligonucleotide with the 5′ to 3′ sequence of TT(C/T)CA(C/T)GA(C/T)TG(C/T)TT(C/T)GT was 5′ end-labelled to high specific activity and used as a probe to isolate peroxidase cDNA clones (Sambrook *et al.*, 1989). Duplicate plaque lifts were made to nylon filters (Amersham), UV-fixed, and pre-hybridized at 36C for 3 h in 6 × SSC, 20 mM Na_2HPO_4 (pH6.8), 5 × Denhardt's, 0.4% SDS, and 500 μg ml^{-1} salmon sperm DNA. Hybridization was in the same buffer, without Denhardt's, at 36C for 16 h. Filters were washed quickly with several changes of 6 × SSC and 0.1% SDS, first at room temperature and finally at 40C, prior to autoradiography for 16 h at –70C with an intensifying screen.

Genomic DNA isolation, library construction, and DNA blot analysis

Soybean genomic DNA was isolated from leaves of greenhouse-grown plants or from etiolated seedlings grown in vermiculite. Plant tissue was frozen in liquid nitrogen and lyophilized before extraction and purification of DNA according to the method of Dellaporta *et al.* (1983). Restriction enzyme digestion of 30 μg DNA, separation on 0.5% agarose gels and blotting to nylon membranes followed standard protocols (Sambrook *et al.*, 1989). For construction of the genomic library, DNA purified from Harosoy 63 leaf tissue was partially digested with *Bam*HI and ligated into the λFIXII vector (Stratagene). Gigapack XL packaging extract (Stratagene) was used to select for inserts of 9 to 22 kb. After library amplification, duplicate plaque lifts were hybridized to cDNA probe.

Blots or filter lifts were prehybridized for 2 h at 65C in 6 × SSC, 5 × Denhardt's, 0.5% SDS, and 100 μg ml^{-1} salmon sperm DNA. Radiolabelled cDNA probe (20–50 ng) was prepared using the Ready-to-Go labelling kit (Pharmacia) and ^{32}P-dCTP (Amersham). Unicorporated ^{32}P-dCTP was removed by spin column chromatography before adding radiolabelled cDNA to the hybridization buffer (identical to pre-hybridization buffer without Denhardt's). Hybridization was for 20 h at 65C. Membranes were washed twice for 15 min at room temperature with 2 × SSC, 0.5% SDS, followed by two 30 min washes at 65C with 0.1 × SSC, 0.5% SDS. Autoradiography was for 20 h at –70C using an intensifying screen and X-OMAT film (Kodak).

RNA blot analysis

For isolation of RNA, tissue was harvested from greenhouse grown plants, dissected, frozen in liquid nitrogen, and lyophilized prior to extraction. Total RNA was purified from seed coats, embryos, pods, leaves and flowers by the standard phenol/chloroform method described by Wang and Vodkin (1994). This method did not afford good yields of RNA from roots, therefore this tissue was extracted with Trizole reagent (Gibco BRL) and total RNA purified according to the manufacturer's instructions with an additional phenol–chloroform extraction step. The amount of RNA was estimated by measuring absorbance at 260 and 280 nm, and by electrophoretic separation in formaldehyde gels followed by staining with ethidium bromide and comparison with known standards. Total RNA (10 μg per sample) was prepared, subject to electrophoresis through a 1% agarose gel containing formaldehyde, and then stained with ethidium bromide to ensure equal loading of samples. The gel was blotted to nylon (Hybond N, Amersham) according to standard methods (Sambrook *et al.*, 1989) and the RNA was fixed to the membrane by UV cross-linking. The preparation of radiolabelled cDNA probe and the conditions of hybridization, washing and visualization by autoradiography were identical to those described for the DNA blot hybridizations.

DNA sequencing

Sequencing of DNA was performed using dye-labelled terminators and Taq-FS DNA polymerase (Perkin-Elmer). The PCR protocol consisted of 25 cycles of a 30 sec melt at 96C, 15 sec annealing at 50C, and 4 min extension at 60C. Samples were analysed on an Applied Biosystems 373A Stretch automated DNA sequencer.

Polymerase chain reaction

PCR amplifications contained 1 ng template DNA, 5 pmol each primer, 1.5 mM $MgCl_2$, 0.15 mM deoxynucleotide triphosphate mix, 10 mM Tris–HCl, 50 mM KCl, pH 8.3, and 1 unit of Taq polymerase (Gibco BRL) in a total volume of 25 μl. Reactions were performed in a Perkin-Elmer 480 thermal cycler. After an initial 2 min denaturation at 94C, there were 35 cycles of 1 min denaturation at 94C, 1 min annealing at 52C, and 2 min extension at 72C. A final 7 min extension at 72C completed the program. The following primers were used for PCR analysis of genomic DNA:

prx2+ CTTCCAAATATCAACTCAAT
prx6-TAAAGTTGGAAAAGAAAGTA
prx9+ ATGCATGCAGGTTTTTCAGT
prx10– TTGCTCGCTTTCTATTGTAT
prx12+ TCTTCGATGCTTCTTTCACC
prx29+ CATAAACAATACGTACGTGAT

Seed coat peroxidase assays

The F_3 seed was measured for peroxidase activity to score the phenotype of the F_2 population because the seed testa is derived from maternal tissue. The seeds were briefly soaked in water and the seed coat was dissected from the embryo and placed in a vial. Ten drops (~500 µl) of 0.5% guaiacol was added and the sample was left to stand for 10 min before adding one drop (~50 µl) of 0.1% H_2O_2. An immediate change in colour of the solution, from clear to red, indicates a positive result and high seed coat peroxidase activity.

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GenBank Accession Number for the soybean *Ep* gene is AFD14502